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Investigations of the genotoxic properties of two synthetic cathinones (3-MMC, 4-MEC) which are used as psychoactive drugs

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Synthetic cathinones (SCAs) are consumed worldwide as psychostimulants and are increasingly marketed as surrogates of classical illicit drugs *via* the internet. The genotoxic properties of most of these drugs have not been investigated. Results of earlier studies show that amphetamines which are structurally closely related to these compounds cause damage to the genetic material. Therefore, we tested the genotoxic properties of two widely consumed SCAs, namely, 3-MMC (2-(methylamino)-1-(3-methylphenyl) propan-1-one) and 4-MEC (2-(ethylamino)-1-(4-methylphenyl) propan-1-one) in a panel of genotoxicity tests. We found no evidence for induction of gene mutations in *Salmonella*/microsome assays, but both drugs caused positive results in the single cell gel electrophoresis (SCGE) assay which detects single and double strand breaks of DNA in a human derived buccal cell line (TR146). 3-MMC induced similar effects as 4-MEC and also caused significant induction of micronuclei which are formed as a consequence of structural and chromosomal aberrations. Negative results obtained in SCGE experiments with lesion specific enzymes (FPG and Endo III) show that these drugs do not cause oxidative damage of DNA. However, moderate induction of TBARS (which leads to the formation of DNA-reactive substances) was observed with 4-MEC, indicating that the drug causes lipid peroxidation while no clear effect was detected with 3-MMC. Results obtained with liver homogenate in SCGE-experiments show that phase I enzymes do not lead to the formation of DNA reactive metabolites. Taken together, our findings indicate that consumption of certain SCAs may cause adverse health effects in users as a consequence of damage to the genetic material.

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Introduction

Cathinones are widely consumed sympathomimetic psychoactive drugs. A natural representative of this group is cathinone, the active principle of khat (*Catha edulis*), which is chewed in Ethiopia, Somalia and several Arabian countries.[1] At the beginning of the 20th century, the first synthetic derivatives were produced and marketed as psychostimulants.[2] In the last decade, synthetic cathinones (SCAs) have been increasingly marketed *via* the internet as so-called "bath salts" as legal alternatives for illicit drugs like cocaine and amphetamine.[2]

Acute poisoning, which includes symptoms such as tachycardia, diaphoresis and hypertension led in some cases to a fatal outcome.[3] As a consequence, specific SCAs (for example mephedrone, 4-MMC) were banned in Western countries but new structural analogues appeared rapidly on the market. Between 2005 and 2011, thirty-four new SCAs were identified in Europe.[4] Among the most widely consumed representatives is at present 4-methylethcathinone (4-MEC) which has been seized in Sweden, Germany, Poland and the US.[5,6] Another relevant structural analog is 3-methylmethcathinone (3-MMC) which is the most frequently consumed SCA in Slovenia[7] and causes severe acute toxic effects in users (Fig. 1).[8]

The neurotoxic and psychostimulant properties of SCAs have been investigated in a number of studies (see for example reviews of Miotto *et al.*, Banks *et al.* and Musselman and Hampton[9–11]) while other toxicological data are limited. About 15 years ago, we found that the consumption of khat causes induction of micronuclei (MN, which are formed as a consequence of structural and/or numerical chromosomal aberrations) in buccal cells of chewers.[12] It is notable that animal experiments indicate that these effects are caused by its active

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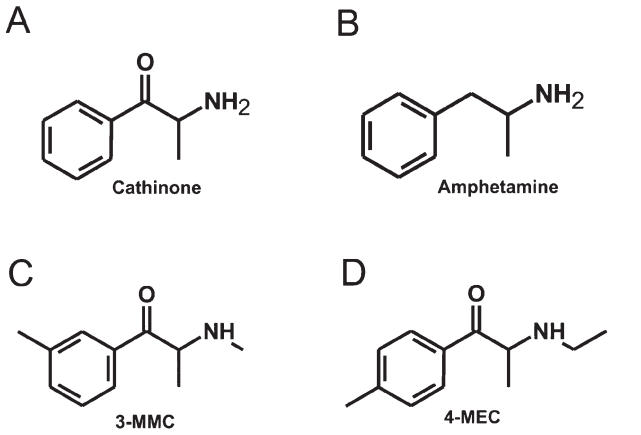

Fig. 1 Chemical structures of the different compounds. A: Cathinone (2-amino-1-phenylpropan-1-one, CAS number: 16735-19-6), B: Amphetamine (1-phenylpropan-2-amine, CAS number: 2706-50-5), C: 3-MMC (2-(methylamino)-1-(3-methylphenyl)propan-1-one, CAS number: 1246816-62-5), D: 4-MEC (2-(ethylamino)-1-(4-methylphenyl) propan-1-one, CAS number: 1266688-86-1).

principle cathinone.[13,14] Also in animal experiments, evidence for clastogenic properties of khat was found.[15,16] In this context, it is also remarkable, that several investigations have been published concerning the DNA damaging properties of amphetamine derivatives.[14] These widely consumed drugs are structurally closely related to cathinones (which are the β-keto analogues of amphetamines).[17]

SCAs have never been studied with regard to their genotoxic properties according to our knowledge. However, their structural relationship to amphetamines and to the natural representative cathinone indicates that they may cause damage to the DNA and stimulated us to investigate the effects of two widely consumed representatives (3-MMC and 4-MEC) in a panel of genotoxicity test systems.

Induction of gene mutations was monitored in *Salmonella*/microsome mutagenicity assay in strains TA98 and TA100 which detect frameshifts and base substituents.[18] We used these strains, since it was found that methamphetamine, which is structurally related to the compounds we tested causes induction of revertants in these indicator strains.[19] Additionally, induction of MN and other nuclear anomalies was monitored in a human derived buccal cell line (TR146) which was chosen since epithelia of the respiratory tract come in direct contact with the drugs. The same cell line was also used in the single cell gel electrophoresis (SCGE) assay, which detects single and double strand breaks and is based on the detection of DNA migration in an electric field.[20] This method was also used to quantify the formation of oxidized DNA bases by use of lesion specific enzymes.[21,22] These later experiments were included, since findings which were obtained with amphetamines suggest that reactive oxygen species (ROS) are involved in the genotoxic activities of these drugs.[19]

To find out if oxidative damage of lipid membranes which leads to the formation of DNA reactive reaction products plays a role,[23] we investigate the impact of the drugs on the formation of thiobarbituric acid reactive substances (TBARS). Furthermore, we conducted additional experiments with liver enzyme homogenate (S9) to find out if the drugs are converted by phase I enzymes to DNA reactive intermediates.

Materials and methods

Chemicals

Low melting point agarose (LMPA) and normal melting point agarose (NMPA) were acquired from Gibco (Paisley, UK). Inorganic salts, 2-aminoanthracene (2-AA), dimethyl sulfoxide (DMSO), propidium iodide, hydrogen peroxide, Triton X-100, Trizma base, trypan blue, fetal calf serum (FCS), bovine serum albumin (BSA), benzo[*a*]pyrene (B[*a*]P), 2-nitrofluorene (2-NF), 4-nitroquinoline-*N*-oxide (4-NQO), mitomycin C, cytochalasin-B, Dulbecco's Phosphate Buffered Saline (DPBS), Dulbecco's modified Eagle Medium (DMEM), L-glutamine and sodium pyruvate were purchased from Sigma-Aldrich (Steinheim, Germany). Aroclor™ 1254-induced rat liver S9 was obtained from Trinova Biochem GmbH (Giessen, Germany). Trypsin-EDTA was ordered from Life Technologies (Karlsruhe, Germany). All chemicals for the stability measurement were purchased from Sigma-Aldrich (Steinheim, Germany).

Test compounds

The two synthetic cathinones namely 2-(ethylamino)-1-(4-methylphenyl)propan-1-one (4-MEC, CAS 1225617-18-4, $C_{12}H_{17}NO$) and 2-(methylamino)-1-(3-methylphenyl)propan-1-one (3-MMC, $C_{11}H_{15}NO$) were obtained as "research chemicals" from China *via* the internet. Identity and purity of the drugs were assessed by GC-MS, ^{1}H NMR and ^{13}C NMR analysis and was >98%. Stock solutions were prepared in DMSO and stored at −20 °C.

Stability measurements

Stock solutions (1.0 mM) were prepared in DMSO; subsequently, test solutions (2.5 μM, 5 μM, 10 μM, 50 μM) were prepared by addition of deionized water. All solutions were stored over a period of 24 h at 37 °C. Aliquots (10 μL) of each concentration were analyzed at 7 different time points (t = 0 h, 2 h, 3 h, 17 h, 20 h, 22 h, 24 h).

After addition of 10 μL of an internal standard (Mephedrone-D3, 1 μg mL^{-1}), the samples were evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted with 100 μL mobile phase A/B (99/1, v/v). Solvent A was water with 0.1% formic acid and 10 mM ammonium formate. Solvent B was methanol with 0.1% formic acid.

The samples (injection volume 1.0 μL) were analyzed using liquid chromatography coupled to a QTrap 4000® tandem mass spectrometer (Sciex, Darmstadt, Germany). Separation was achieved using a Synergi Polar-RP column (100 mm × 2 mm, 2.5 μm) with an equivalent guard column (4 mm × 2 mm; Phenomenex, Aschaffenburg, Germany). Column oven temperature was set at 40 °C, gradient elution was performed

using the following gradient: 0–4 min: 1% B–13% B; 4–10 min: 13% B–50% B; 10–12 min: 50% B–95% B; 12–14 min: 95% B–1% B; 14–17 min 1% B. The flow rate was in all experiments 0.4 mL min^{-1}. Isopropanol was added post-column with a flow rate of 0.1 mL min^{-1}.

Salmonella/microsome assays

The test compounds were analyzed in Ames microplate format (MPF) assays according to the instructions of the manufacturer (Ames MPF™, Xenometrix AG, Allschwil, Switzerland; see also Fluckiger-Isler and Kamber).[24] This procedure differs from the plate incorporation protocol as it is performed in liquid media with 384-well microplates. The experiments were conducted with the *Salmonella typhimurium* strains TA98 (*his*D3052, *rfa*, Δ*bio*, *uvr*B, pKM101) and TA100 (*his*G46, *rfa*, Δ*bio*, *uvr*B, pKM101). TA100 detects base pair substitutions while TA98 is sensitive towards mutagens which cause frameshifts.[18]

Different concentrations of the drugs (0.01, 0.1 and 1.0 mM) were tested in the presence and absence of a metabolic activation mix (S9). 4-Nitroquinoline-*N*-oxide (4-NQO, 0.1 μg mL^{-1}) and 2-nitrofluorene (2-NF, 0.4 μg mL^{-1}) were used as positive controls in assays without metabolic activation. 2-Aminoanthracene (2-AA, 5.0 μg mL^{-1}) was used as positive control in experiments with S-9 mix. Stock solutions of the test compounds were prepared with DMSO which was also added to the control cultures according to the instructions of the manufacturer (Ames MPF™, Xenometrix AG, Allschwil, Switzerland).

Mutagenic effects were determined by measuring changes of the color of the wells from purple to yellow. The number of wells which contained his^+ revertants were counted and compared with the solvent control. Each concentration was tested in triplicate.

Cultivation of human cell lines (TR146)

The human cell line TR146 is derived from buccal epithelial tissue.[25] The cells were a gift from J. G. Rheinwald (Dermatology Institute of Boston, MA, USA). They were cultured under standard conditions (37 °C, humidified atmosphere, 5% CO_2) in DMEM which was supplemented with 10% FCS. The cell line was stored in liquid nitrogen and after re-cultivation, the 4^{th} to 6^{th} passage from stock cultures was used for the experiments. The media were changed every 2–3 days and when the cultures had reached confluency, the cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS), detached with Trypsin/EDTA, centrifuged and sub-cultured.

Single cell gel electrophoresis (SCGE) assays (standard conditions)

The SCGE assays were conducted under standard alkaline conditions.[20] The indicator cells were cultivated overnight in regular 6-well plates, in 3.0 mL DMEM with 10% FCS per well. Subsequently, the medium was changed and aqueous solutions of the drugs (total volume 3 mL) were added to reach final concentrations (5–50 μM). The cells were exposed to the drugs for 3 h and 24 h. H_2O_2 (50 μM) was used in all experiments as a positive control.

Solutions of the drugs were prepared from deep frozen stock before each experiment and further dissolved in serum-free medium. After incubation in the dark, the cells were washed two times with DMEM (containing 10% FCS) and centrifuged (200*g*, 8 min, 16 °C). After centrifugation, the cytotoxicity of the test compounds was determined with the trypan blue exclusion test,[26] then the cells were re-suspended in low melting agarose (0.5% LMPA). Afterwards, the cells were spread on pre-coated agarose slides (1.5% NMPA) and lysed in the dark at 4 °C for at least 60 min. After 30 min unwinding under alkaline conditions (pH > 13), electrophoresis was carried out for 30 min (300 mA, 25 V) and neutralization was performed twice for 8 min. Air-dried slides were stained with propidium iodide (10 μg mL^{-1}); subsequently the percentage of DNA in the tails was measured by use of a computer aided image analysis system (Comet IV, Perceptive Instruments Ltd, Burry St Edmunds, UK). Each experimental point was performed as a triplicate, 3 slides were made from each culture and 50 cells were evaluated per slide.

Additional SCGE experiments were performed with exogenous liver homogenate (S9 mix) which was prepared according to the standard recipe of Maron and Ames.[18] The cultures were treated with the drugs (for 3 h) as described above. Additionally, S9 mix was added (final value 4%) according to the OECD guideline 487.[27] Benzo[*a*]pyrene (B[*a*]P, 50 μM which was diluted in DMSO) was used as a positive control in experiments with S9. In further experimental series, heat inactivated S9 (HS9, heated to 50 °C for 10 min) and BSA were added to the incubation mixtures instead of active S9 to find out if the drugs are inactivated *via* protein binding effects.

Single cell gel electrophoresis (SCGE) assays with lesion-specific enzymes

To determine the impact of the drugs on formation of oxidatively damaged purines and pyrimidines, additional experiments with lesion specific enzymes (formamidopyrimidine DNA glycosylase, FPG and endonuclease III, Endo III) were performed. The measurements were conducted according to the protocol of Collins and Dušinská.[28] The cells (TR146) were treated with the test compounds as described above. To establish the optimal amounts of the enzymes, calibration experiments were performed before the main experiments (data not shown) according to the protocol of Collins *et al.*[29]

After lysis, the slides were washed twice with enzyme reaction buffer (pH 8.0) for 8 min. Subsequently, the nuclei were treated with 50 μL of the enzyme solutions or with the enzyme buffer. The incubation time for FPG was 30 min and for Endo III, 45 min at 37 °C respectively. After the treatment and 30 min unwinding under alkaline conditions (pH > 13), electrophoresis was carried out (30 min, 300 mA, 1.0 V cm^{-1}, at 4 °C, pH > 13). Subsequently, the slides were processed and evaluated as described above. The experiments were performed in triplicate, three parallel cultures were made and from each culture three slides were prepared and 50 cells were evaluated

from each slide. The values which were obtained with the enzyme buffers were subtracted from the results which were obtained with the enzyme solutions.

Measurement of thiobarbituric acid reactive substances (TBARS)

For the TBARS measurement, cells were cultivated and treated with the drugs 3-MMC and 4-MEC (for 3 h) as described for the SCGE experiments. For each experimental point 4×10^6 were treated as described by Lieners *et al.*[30] and the reference protein in various homogenates and cytosols was determined according to the Bradford/Biorad procedure.[31] Subsequently, the chromogen (TBARS) was quantified by spectrophotometric reading at 532 nm. The amount of TBARS was expressed as nmol per mg protein per mL (for details see Huber *et al.*[32]).

Cytokinesis-block micronucleus (CBMN) assays with TR146 cells

The CBMN experiments with TR146 was performed as described in detail in the paper of Koller *et al.*[33] Briefly, the cells were seeded in 6-well plates and allowed to attach overnight. Afterwards, the medium was removed and the cells were washed with DPBS, then they were treated with different concentrations of the test compounds in serum-free medium for 24 h. Mitomycin C (1.0 μg mL^{-1}) was used as a positive control, medium with DMSO (1%) was used in the control cultures. After exposure of the cells to the drugs and washing with PBS, they were incubated with cytochalasin B (3.0 μg mL^{-1}) and DMEM (with 10% FCS) for another 24 h. Subsequently, the cells were washed again, trypsinized and processed as described in the paper of Koller *et al.*[33]

Two cultures were made from each experimental point and from each culture 1000 cells were evaluated. Different endpoints were scored, namely mono-nucleated, bi-nucleated (BN) and multi-nucleated cells, as well as, the rates of MN, MN in BN-cells (BN–MN), nuclear buds (Nbuds), nucleoplasmatic bridges (NPBs), apoptosis and necrosis. The cytokinesis-block proliferation indices (CBPI) were calculated with 500 cells according to the formula $CBPI = [M1 + 2M2 + 3(M3 + M4)]/N$ (N is the total number of scored cells, M1–M4 refers to the number of cells with one to four nuclei). The toxicity of the compounds was determined indirectly by the assumption that

Table 1 Results of the stability experiment[a]

Compounds	Concentration μM	Incubation time (h)					
		0	2	3	17	22	24
3-MMC (area ratio 3-MMC/D3-mephedrone)	2.5	0.82	0.68	0.81	0.78	0.87	0.85
	5	1.67	1.48	1.38	1.64	1.38	1.54
	10	3.31	3.29	2.59	3.18	2.77	3.55
	50	15.85	13.05	16.35	15.40	14.05	19.10
4-MEC (area ratio 4-MEC/D3-mephedrone)	2.5	0.70	0.67	0.73	0.79	0.81	0.79
	5	1.51	1.16	1.20	1.55	1.23	1.42
	10	2.84	2.05	2.37	2.86	2.26	3.33
	50	13.75	12.75	14.15	14.20	13.35	15.85

[a] Values indicate means of two measurements.

Table 2 Results of gene-mutations assays obtained with the bacterial indicator strains TA98 and TA100 in the presence and absence of metabolic activation mix[a]

Compounds	Concentration mM	TA98 − S9 Mean ± SD	TA98 + S9 Mean ± SD	TA100 − S9 Mean ± SD	TA100 + S9 Mean ± SD
Neg. ctrl (DMSO)[b]	0.0	0.7 ± 0.6	1.3 ± 1.2	8.0 ± 1.0	6.3 ± 2.3
3-MMC	0.01	2.0 ± 1.0	1.0 ± 1.7	6.0 ± 2.6	6.3 ± 2.3
	0.10	0.7 ± 0.6	1.3 ± 1.5	6.0 ± 3.6	5.3 ± 2.3
	1.00	2.0 ± 1.0	1.7 ± 0.6	6.3 ± 4.2	7.7 ± 1.5
4-MEC	0.01	0.7 ± 0.6	1.7 ± 1.5	9.3 ± 4.0	6.7 ± 1.5
	0.10	1.0 ± 1.0	2.3 ± 1.2	7.3 ± 2.5	7.7 ± 3.2
	1.00	1.7 ± 0.6	1.7 ± 1.2	6.7 ± 1.5	11.0 ± 2.6
Pos. ctrl[b]		**41.7 ± 1.5***	**48.0 ± 0.9***	**48.0 ± 1.2***	**34.7 ± 0.6***

[a] The *Salmonella typhimurium* strains TA100 and TA98 were exposed to different concentrations of two SCAs in the presence and absence of metabolic activation mix (rat liver S9) as described in Materials and methods. Numbers indicate means ± SD of results obtained in three parallel experiments; asterisks indicate values (bold) which are significantly different from those found in the respective controls ($p < 0.05$). However, only those were considered as mutagenic, if fold induction of the revertant number was greater than two-fold over the baseline. [b] Neg. ctrl – solvent control (1% DMSO); Pos. ctrl – positive controls without S9: 4-NQO (0.1 μg mL^{-1}) and 2-NF (0.4 μg mL^{-1}); with S9: 2-AA (5.0 μg mL^{-1}); 1% DMSO was used as a negative control.

a CBPI of 1.0 corresponds to 100% cytotoxicity. Five concentrations of each drug were used to determine the CBPI values. In agreement with OECD guideline 487,[27] for evaluating the formation of MN and other nuclear anomalies only concentrations which caused less than 60% cytotoxicity were included.

Statistical analyses

The statistical evaluation of all results was performed with the GraphPad Prism 5 Project software system (San Diego, CA, USA).

The results of the bacterial tests, SCGE experiments and MN assays are presented as means ± SD.

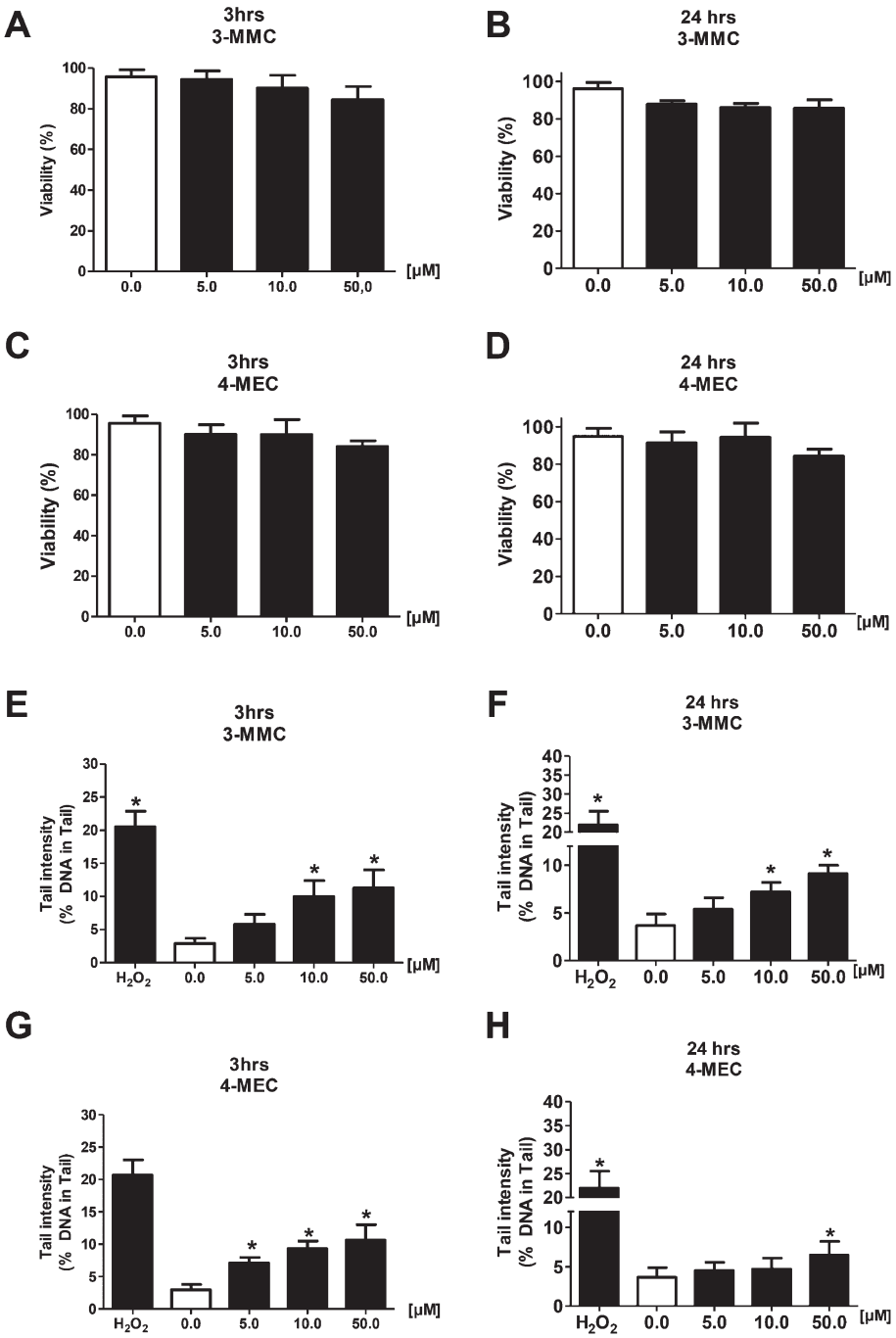

Fig. 2 Cytotoxic and genotoxic properties of 3-MMC and 4-MEC in TR146 cells. The indicator cells were exposed to different concentrations of the test compounds for 3 and 24 hours. Subsequently, the cytotoxic activities of the drugs were monitored with trypan blue (A–D) and comet formation was measured after electrophoresis (E–H, for details see Materials and methods). Bars indicate means ± SD of data obtained with three parallel cultures per experimental point (from each culture 3 slides were made and 50 cells were evaluated per slide). Stars indicate statistical significance ($p \leq 0.05$, ANOVA).

The CBMN assay results were analyzed with the Kruskal–Wallis test followed by the Dunn's test. The results of SCGE experiments were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test as suggested by Lovell and Omori.[34] *P*-Values ≤0.05 were considered as statistically significant.

Results of Ames MPF assays were analyzed according to the criteria for positive results as defined by Fluckiger-Isler and Kamber.[24] Briefly, the mean numbers of positive (yellow) wells per concentration were calculated from triplicates and the fold-increase above the baseline (mean of negative control plus SD) was determined for each concentration (for further details see Fluckiger-Isler and Kamber[24]). A two-fold increase compared to the control value was considered as a positive result.

Results

Stability of the test compounds

The stability of the aqueous solution of the test compounds (which have been pre-dissolved in DMSO) was determined by liquid chromatography tandem mass spectrometry (LC-ESI-MS/MS). The results are shown in Table 1. It can be seen that the compounds were (relativity) stable over a period of 24 h.

Salmonella/microsome assays

The results obtained in the bacterial mutagenicity experiments are summarized in Table 2. It can be seen that no indication for induction of gene mutations was detected in strains TA98 and TA100 in the presence and absence of the metabolic activation mix (S9).

Single cell gel electrophoresis experiments (standard conditions) and genotoxicity test

Fig. 2A–H depict the results of SCGE experiments. In all experimental series, the cytotoxic activities of the drugs were monitored with trypan blue as it is known that they may cause misleading results in comet assays.[20] It can be seen, that moderate effects were observed with both drugs when the cells were exposed to concentrations ≥50 μM (Fig. 2A–H). Therefore, higher concentrations were not tested.

Both SCAs induced concentration-dependent DNA migration in the buccal derived cell line after 3 h exposure. It can be seen in Fig. 2A–H that 3-MMC caused a similar effect as 4-MEC; the LOEL of the latter drug was 5.0 μM, with 3-MMC it was 10.0 μM. Extension of the exposure time to 24 h lead to a moderate (non-significant) decrease of the genotoxic effects of both compounds. H_2O_2 was used as a positive control and caused clear effects in all experiments.

Fig. 3 shows the results which were obtained after the cells were exposed to the drugs in the presence and absence of the exogenous activation mix S9. Additionally, cultures were treated with HS9 and BSA (the protein levels in the cultures were identical to the concentrations which were present in the cultures which were exposed to active S9). It can be seen that the drugs caused significant induction of DNA migration in the absence of S9 (as in the previous experiments). Addition of liver enzyme mix caused no increase of DNA migration but a slight reduction by 16.7% and 21.93% in tests with 3-MMC and 4-MEC respectively. Similar effects were observed with HS9 and also with BSA.

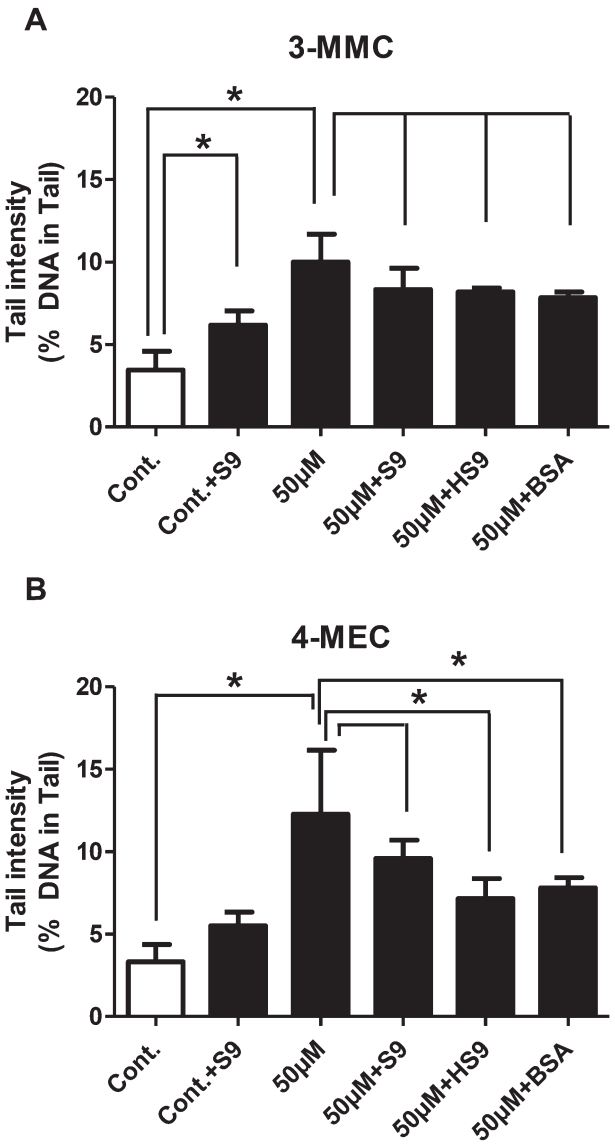

Fig. 3 Impact of rat liver S9, bovine serum albumin (BSA) and heat inactivated S9 (HS9) on comet formation induced by 3-MMC and 4-MEC in TR146. The cells were treated with solutions of the drugs (50 μM 3-MMC or 4-MEC) with and without S9 mix, HS9 or BSA (final protein concentration 0.3 mg mL^{-1}). B[*a*]P (50 μM) was used as a positive control and induced a significant effect (with S9 the tail intensity was 21.6 ± 4.6 and without S9 9.6 ± 2.5). After treatment, DNA migration was determined in SCGE experiments under standard conditions. Bars represent means ± SD of results obtained with tree cultures in parallel. From each culture, three slides were made and 50 cells were analyzed for comet formation per slide.

Results of SCGE experiments with lesion specific enzymes

The findings which were obtained in experiments with FPG and Endo III are summarized in Fig. 4A–D. The bars show the extent of DNA migration after treatment of the nuclei with the enzymes (buffer values were subtracted) which is attributable to formation of oxidized purines and pyrimidines. It can be seen, that no indication of formation of oxidatively damaged bases was found in these experiments.

TBARS measurements

The results are shown in Fig. 5. It can be seen that 4-MEC caused a moderate but significant increase of TBARS formation while no clear effect was observed with 3-MMC under identical experimental conditions.

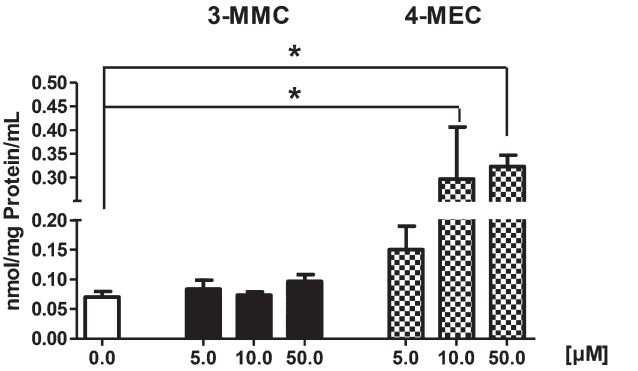

Fig. 5 Impact of the drugs on formation of TBARs. The cells were exposed to 3-MMC or 4-MEC for 3 h. Subsequently, they were homogenised and TBARS concentrations were determined spectrophotometrically. Bars indicate means ± SD of results obtained with three cultures per experimental point. Stars indicate statistical significance ($p \leq 0.05$, ANOVA).

Impact of drugs on the induction of micronuclei and other nuclear anomalies

Table 3 summarizes the results of the MN cytome assays. It can be seen, that 3-MMC caused a concentration-related increase of the MN-rates in binucleated cells, while no effect was observed with 4-MEC under identical experimental conditions. Both drugs also caused a moderate increase of the rates of nuclear buds (Nbuds) and of nucleoplasmic bridges (NPBs). Also the frequencies of apoptotic and necrotic cells were increased in cultures which were exposed to the drugs, but none of these effects reached statistical significance.

Discussion

Taken together, our findings indicate that both drugs which are widely used as psychostimulants do not cause gene mutations in bacteria but clear evidence for increased comet formation was found in a human derived buccal cell line (TR146). Only one of the compounds, namely 3-MMC, induced a significant increase of MN formation at the highest concentrations in the cytokinesis-block assay.

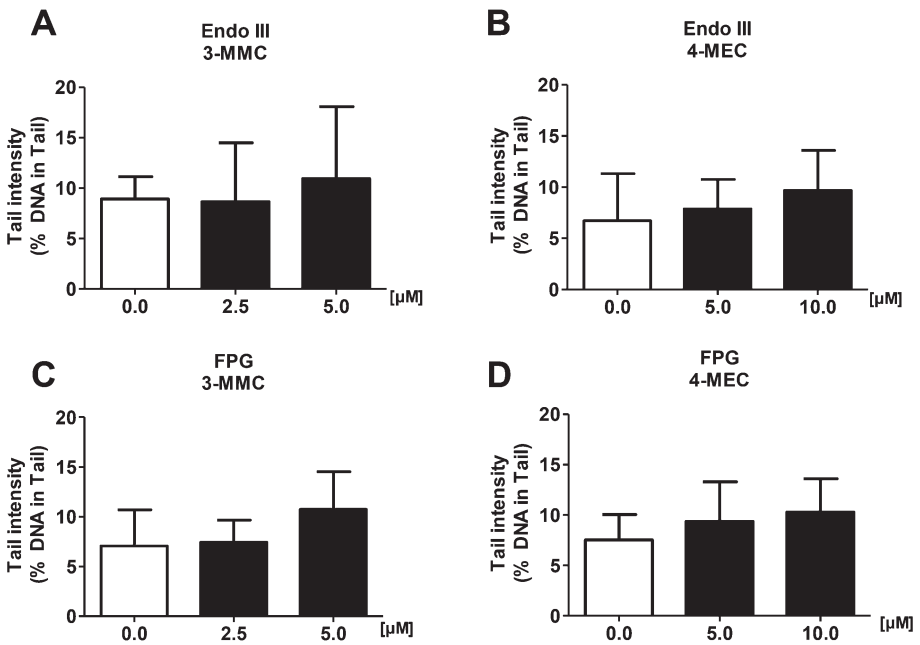

Fig. 4 Impact of 3-MMC and 4-MEC on formation of oxidatively damaged pyrimidines (Endo III sensitive sites, Fig. 3A and B) and purines (FPG sensitive sites, Fig. 3C and D) in a human derived buccal cell line (TR146). The cells were exposed to the drugs for 24 h. Subsequently, the nuclei were isolated and treated with enzymes or with the buffers only as described in Materials and methods. Subsequently, DNA migration was monitored. Bars indicate means ± SD of results obtained with three cultures per experimental point (values obtained with the enzyme buffers were subtracted from values obtained with the enzymes). From each culture, three slides were made and 50 cells were evaluated per slide.

Table 3 Impact of two different synthetic cathinones (SCAs) on nuclear anomalies in a human-derived buccal epithelial cell line (TR146)[a]

Compounds	Concentrations μM	CBPI Mean ± SD	CT %	BN-MNi[b] Mean (‰) ± SD	MNi[c] Mean (‰) ± SD	Nbuds Mean (‰) ± SD	NPBs Mean (‰) ± SD	Apoptosis Mean (‰) ± SD	Necrosis Mean (‰) ± SD
Neg. ctrl (DMSO)	0 μM	2.05 ± 0.52	—	1.89 ± 0.74	1.93 ± 0.17	1.69 ± 0.81	1.56 ± 0.51	0.99 ± 0.08	1.00 ± 0.19
3-MMC	25	2.09 ± 1.21	0.95	2.98 ± 1.56	2.98 ± 1.56	1.71 ± 0.11	1.62 ± 0.93	1.12 ± 0.91	1.12 ± 0.53
	50	2.12 ± 1.10	3.8	3.12 ± 0.98	4.58 ± 1.23	1.98 ± 1.12	1.55 ± 1.12	1.28 ± 0.54	1.62 ± 0.91
	75	1.89 ± 0.78	15.2	3.48 ± 1.60	4.49 ± 1.50	1.92 ± 0.93	1.93 ± 0.81	1.11 ± 0.12	1.39 ± 0.33
	100	**1.69 ± 0.31***	34.2	**4.22 ± 0.39***	**6.99 ± 0.84***	2.23 ± 1.12	2.09 ± 1.11	1.39 ± 1.12	2.38 ± 0.41
	150	**1.41 ± 0.22***	60.2	**4.82 ± 0.55***	**7.49 ± 0.99***	2.69 ± 1.31	2.39 ± 0.92	1.44 ± 0.83	**4.29 ± 0.89***
4-MEC	25	2.00 ± 0.54	4.8	2.89 ± 1.23	2.89 ± 1.23	1.90 ± 0.52	1.45 ± 1.10	0.91 ± 0.10	1.49 ± 0.70
	50	1.80 ± 0.98	23.8	2.38 ± 0.99	3.98 ± 0.98	1.78 ± 1.13	1.68 ± 0.91	0.95 ± 0.61	1.30 ± 1.12
	75	1.67 ± 0.43	36.2	1.99 ± 1.79	2.72 ± 1.90	2.39 ± 0.70	1.59 ± 0.93	1.39 ± 0.90	1.46 ± 0.97
	100	**1.20 ± 0.54***	81	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	150	**1.12 ± 0.63***	88.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pos. ctrl	1 μg mL^{-1}	1.73 ± 0.19	31	**40.50 ± 3.41***	**53.59 ± 12.31***	**9.93 ± 0.91***	**4.54 ± 1.21***	**2.16 ± 0.91***	**4.91 ±.0.79***

[a] Cells were treated with 5 different concentrations of the SCAs (25, 50, 75, 100, 150 μM) for 24 h. Subsequently, the cells were washed with PBS and incubated for further 48 h with cytochalasin B (3.0 μg mL^{-1}). The cells were harvested by trypsinization and slides were prepared by cytocentrifugation as described in the OECD guidelines.[27] Numbers represent results (means ± SD) obtained with duplicate cultures. Per experimental point, two slides were prepared and 1000 cells were evaluated from each slide. *Asterisks indicate significant differences from negative control values (Kruskal–Wallis test followed by Dunns's test, $p \leq 0.05$). n.d. – not determined (severe inhibition of cell division). [b] Number of binucleated cells with micronuclei. [c] Total number of MN. Abbreviations: BN-MNi – binucleated cells with micronuclei; CBPI – cytokinesis-block proliferation index; CT – cytostasis (%); MNi – total number of micronuclei; Nbuds – nuclear buds; NPBs – nucleoplasmic bridges; Neg. ctrl – solvent control (1% DMSO); Pos. ctrl – mitomycin C (1.0 μg mL^{-1}).

The lack of a positive result obtained with 4-MEC is due to a pronounced decrease of the mitotic activity of the cells. As shown in Table 3, it was not possible to evaluate the MN formation by this drug at concentrations >75 μM, while 3-MMC caused less pronounced inhibition of cell division and induced significant MN formation at levels of 100 μM and 150 μM.

According to our knowledge, this is the first investigation concerning the genotoxic properties of SCAs. As mentioned in the introduction, results of earlier experiments indicate that cathinone, which is the active principle of khat and khat extract cause chromosomal breaks and/or MN in mice (for review see Al-Habori[35]). This natural representative has not been studied *in vitro* with mammalian cell lines but some data are available which concern induction of DNA damage by amphetamines, which are structurally closely related to the cathinones. For example, clear positive results were obtained with methamphetamine in the absence of the metabolic activation mix in MN experiments with a Chinese hamster ovary (CHO) cell line,[19] but the concentrations which were required to cause an effect (≥2 mM) were substantially higher than the concentration of 3-MMC which caused positive results in the present experiments.[19] The same drug was also tested in the *Salmonella*/microsome assay and clear induction of his$^+$ revertants was found in strains TA98 and TA100 in the absence of the exogenous activation mix (S9). When S9 was added to the incubation mixtures, the genotoxic effects of the drug disappeared; identical effects were also observed in MN experiments.[19] To find out if 3-MMC and 4-MEC are activated by phase I enzymes we conducted additional experiments with exogenous liver enzyme homogenate (S9). We found no evidence of increased DNA damage in the presence of S9, however, a moderate decrease of comet formation was observed (Fig. 3). A moderate reduction of comet formation was also observed with HS9 and BSA, indicating that protein binding effects are involved.

It has been postulated that the DNA damaging properties of amphetamines are due to oxidative damage of the genetic material.[19,36–38] Furthermore, it was stressed on the basis of animal experiments that khat causes oxidative damage as well, since decreased serum levels of radical scavenging enzymes were found after administration of extracts in animal experiments.[39] However, in this context it is notable, that no evidence for oxidative DNA damage was found in a study with khat chewers.[40] The fact that we found no evidence of formation of oxidatively damaged DNA bases in the present experiments indicates that SCAs cause damage to the genetic material *via* other molecular mechanisms. In the case of 4-MEC (but not 3-MMC) we found clear evidence for significant formation of TBARS, this observation indicates that reaction products of the lipid peroxidation chain reaction may account (at least partly) for the DNA-damaging effects of the former drug.

As mentioned in the introduction, it is known that MN are formed as a consequence of structural and chromosomal aberrations and it was shown that increased MN-rates in human buccal cells are indicative of elevated cancer risk.[41] The serum level of 4-MEC which were detected in blood samples of drug users are in the range between 46 and 152 ng mL^{-1}.[42] For 3-MMC, we could not find any data but the levels of cathinone were determined in several investigations in khat chewers and were similar to those detected with 4-MMC which is structurally closely related to 3-MMC.[43,44] It is notable, that these concentrations are substantially (2–3 orders of magnitude) lower

than the levels of the two SCAs which caused significant DNA damage in the present study. However, it cannot be ruled out that exposure of the epithelia of the respiratory tract (in particular in the nasal and mouth mucosa) is substantially higher and causes DNA damage in cells which come into direct contact with the SCAs.

Since our results indicate that SCAs cause damage to the genetic material, which may lead to adverse health effects in humans in particular, to cancer.[45,46] In this context, it is notable, that the consumption of khat, which causes formation of MN in buccal cells[12] and chromosomal damage in animals has been postulated to lead to premalignant lesions and consequently, also to oral cancer (for reviews see Soufi *et al.* and Al-Habori[35,47]). However, the exposure situation of khat chewers is different from that of consumers of synthetic cathinones, therefore, further experiments should be conducted with laboratory rodents and users to assess the genotoxic properties of these drugs which are increasingly marketed worldwide.

Conflict of interest

The authors state to have no conflict of interest.

Abbreviations

2-AA	2-Aminoanthracene
B[*a*]P	Benzo[*a*]pyrene
BN-MN	Binucleated cells with micronucleus
BSA	Bovine serum albumin
CBMN	Cytokinesis-block micronucleus assay
CBPI	Cytokinesis-block proliferation index
CT	Cytostasis
HS9	Heat inactivated S9
4-MEC	2-(Ethylamino)-1-(4-methylphenyl)propan-1-one
3-MMC	2-(Methylamino)-1-(3-methylphenyl) propan-1-one
2-NF	2-Nitrofluorene
MN	Micronucleus
Nbuds	Nuclear buds
4-NQO	4-Nitroquinoline-*N*-oxide
NPBs	Nucleoplasmatic bridges
SCGE	Single cell gel electrophoresis
SCAs	Synthetic cathinones
TBARS	Thiobarbituric acid reactive substances

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